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Characterisation of the diol dehydratase *pdu* operon of *Lactobacillus collinoides*

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Abstract

The three genes (*pduCDE*) encoding the diol dehydratase of *Lactobacillus collinoides* were sequenced. They exhibited strong identities with the *ddrABC* and *pduCDE* genes of *Klebsiella oxytoca* and *Salmonella enterica*, respectively. These genes are part of a putative operon with at least four other genes. An eighth open reading frame was identified as homologous to the *pocR* gene (encoding the operon regulatory protein). Although the enzyme was detected in exponential growth phase, *PduCDE* activity was increased at the end of exponential phase in presence of 1,2-propanediol.

Keywords: Diol dehydratase; *pdu* operon; *Lactobacillus collinoides*

1. Introduction

Few micro-organisms have the ability to use glycerol in a reductive branch. In this degradation pathway, it is converted in a two-step process to 1,3-propanediol (1,3-PD) excreted into the extracellular medium [1]. The first reaction involves the transformation of glycerol into 3-hydroxypropionaldehyde (3-HPA) by a coenzyme B-12-dependent dehydratase enzyme. The second reaction involves the dismutation of the aldehyde by an NADH-linked reductase producing 1,3-PD. Two isofunctional enzymes, glycerol dehydratase (EC 4.2.1.30) and diol dehydratase (EC 4.2.1.28) catalyse the reaction of glycerol dehydratation to 3-HPA as well as the reactions of 1,2-PD to propionaldehyde and ethanediol to acetaldehyde [2]. The glycerol/diol dehydratase genes of *Clostridium pasteurianum* and of enteric bacteria such as *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Citrobacter freundii* and *Salmonella enterica* serovar *typhimurium* have been cloned and sequenced. These enzymes exhibit a $\alpha_2\beta_2\gamma_2$ composition where α, β and γ are the large, medium and small subunits, respectively, encoded by three contiguous open reading frames (ORFs) [3]. Glycerol dehydratase has greater specificity for glycerol and affinity for the coenzyme B-12 than diol dehydratase [4]. Glycerol dehydratase of *K. pneumoniae* and *C. freundii* form part of the *dha* regulon induced under anaerobic conditions by glycerol and dihydroxyacetone. The diol dehydratase genes of *S. enterica* are part of the *pdu* operon in which regulation is mediated by PocR, a transcriptional regulator of the AraC family [5].

The anaerobic degradation of glycerol into 1,3-PD has been reported for a long time in the genera *Lactobacillus* [1]. Bacteria from this genera lack the oxidative pathway of glycerol degradation, hence glycerol can not be metabolised as a sole carbon source. Glycerol utilisation results from the necessity to recycle, via the 1,3 PD dehydrogenase, the NADH formed during the hexose catabolism [6]. The glycerol dehydratase of *Lactobacillus reuteri* has been purified and characterised but its gene has not been sequenced yet. Although it has a similar molecular mass compared to the other dehydratases studied, it is composed of four identical subunits of 52 kDa each [7].

Lactobacillus collinoides is a heterofermentative lactic acid bacterium commonly found in cider [8]. Its presence in cider has been associated to an alteration known as 'piqûre acroléique' leading to the formation of acrolein (2-propenal), a lachrymatory chemical generating a 'pep-

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pery' flavour [9]. Acrolein is not issued from the bacterial metabolism but is rather in chemical equilibrium with 3-HPA. The aldehyde can spontaneously form acrolein by thermal dehydratation, spoiling the quality of the cider.

Recently, a 150-bp DNA fragment exhibiting strong homologies with the gene encoding the large subunit of glycerol dehydratase from *C. freundii*, *K. pneumoniae* and *C. pasteurianum* was cloned and sequenced [10]. Here, we report the nucleotide sequence and the characterisation of the distal region of the putative operon encoding the diol dehydratase genes of *L. collinoides* as well as the conditions of its expression.

2. Materials and methods

2.1. Bacterial strains and growth conditions

This study was performed with *L. collinoides* LMG 18850, isolated from French cider. Cultures were grown at 30°C without shaking in MRS medium [11] supplemented with 2% (w/v) glucose unless when specified.

2.2. Chromosomal DNA extraction and PCR amplification

Cells were harvested from 20 ml culture by centrifugation and DNA was extracted by the NUCLEBOND® AX kit (Macherey-Nagel). The nucleotide sequence was obtained by a ligase-mediated strategy as previously described [12] using the plasmid pBluescript SK+ (Stratagene) as vector. Restriction endonucleases, alkaline phosphatase and T4 DNA ligase were obtained from Roche (Mannheim, Germany) and used according to the manufacturer's instructions. PCR were carried out in a 25-μl volume using Ready To Go PCR beads (Pharmacia Biotech) with oligonucleotides deduced from the diol dehydratase sequence of *L. collinoides* and the reverse primer of the vector. PCR products were purified using the QIAquick kit (Qiagen).

2.3. DNA sequencing, sequence analysis and accession number

Sequencing of PCR fragments was carried out using the dideoxy chain-termination method [14] with the ABI Prism sequencing system (PE Biosystem). DNA sequences were analysed using the Mac Vector (Kodak, Scientific imaging Systems) programme. Database searches were performed with the BLAST programme [13] and alignments were performed with the VectorNTI programme (InforMax Inc.) The EMBL accession number for the sequence is AJ297723.

2.4. RT-PCR experiments

Total RNA was isolated from exponentially growing cells by using the RNeasy Midi Kit (Qiagen, Santa Clara, CA, USA). For reverse transcriptions 10 μg of the RNA preparation was treated for 30 min at 37°C with 20 U of RNase-free DNase I (Roche, Mannheim, Germany). Reverse transcriptase reactions were performed at 55°C using the RACE 5′/3′ kit (Roche) with 12.5 pmol of primer RT1 (5′-GCTTCAAGGTAAAGCATTGATTTACC-3′) (Fig. 1A) and 2 μg RNA. The cDNA were then purified with the QIAquick kit (Qiagen). RT-PCR was performed using Ready To Go PCR beads (Pharmacia Biotech), one tenth of the purified cDNA as template and 20 pmol of primers (RT2: 5′-CCTGAAGTAAACCGCATC-3′; RT3: 5′-TGGATAACTTGAGTCGGAC-3′; RT4: 5′-CTGTTGATGCAGGTGTTC-3′). Absence of contaminating genomic DNA was controlled by non-reverse-transcribed PCR performed under the same conditions, excepted that AMV reverse transcriptase was replaced by H_2O.

2.5. Southern blot hybridisation

Transfer of digested DNA onto Hybond-N+ membranes (Amersham, Little Chalfont, UK) and hybridisation were carried out as described by Sambrook et al. [14]. The DNA fragment used as probe was amplified by PCR from genomic DNA of *L. collinoides* with the primers S1 (5′-GGTAAATCAATGCTTTACCTTGAAGC-3′) and S2 (5′-TAATTGGCATGATACCAC-3′).

2.6. Diol dehydratase assay and metabolites determination

The activity of diol dehydratase was determined by the MBTH method [3]. The 3-HPA was determined with the colourimetric tryptophan method [15]. Glycerol was measured by high-performance liquid chromatography using a Waters™ 600 Module with an HPX-87H column (Bio-Rad, Hercules, CA, USA) heated at 35°C and a refractometer detector (LKB Germany). The eluant was 5 mM H_2SO_4 with a flow rate of 0.5 ml min^{-1}.

3. Results

3.1. Nucleotide sequence of the genes of the diol dehydratase and surrounding regions

A total sequence of 7291 bp was obtained by LM-PCR from the initial 150-bp fragment [10]. Analysis revealed the presence of at least eight ORFs (named *pduABCDEGH* and *pocR*). The restriction map and the apparent genetic organisation of the diol dehydratase region are depicted in Fig. 1. Among the ORFs deduced from the nucleotide sequence, three (*pduCDE*) presented strong homologies with the genes of diol dehydratase of *K. oxytoca*, *S. enterica* and *K. pneumoniae* (Table 1). These genes (*pduCDE*) encode polypeptides of 558, 230 and 172 amino acids with

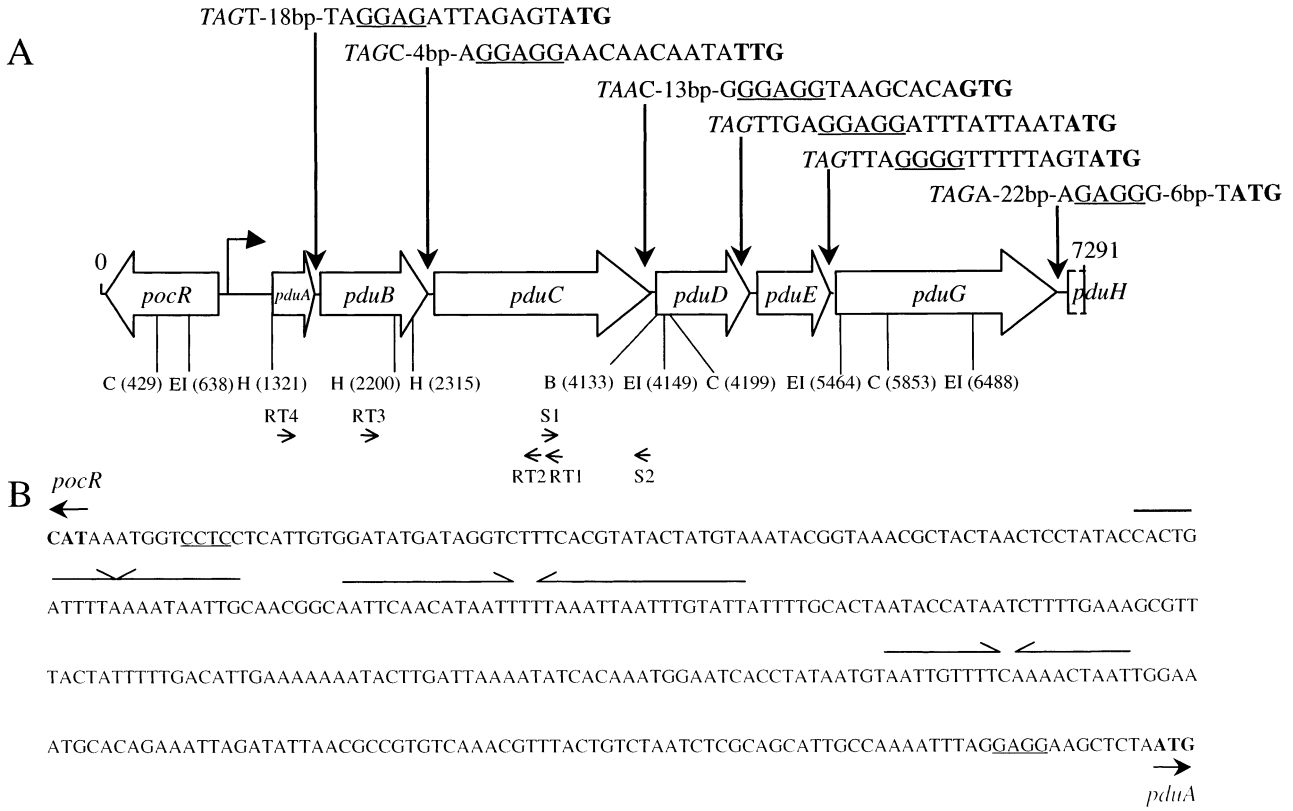

Fig. 1. A: Genetic organisation of the partial *pdu* operon of *L. collinoides*. Positions of enzymes used for the LM-PCR (H: *Hin*dIII, C: *Cla*I, EI: *Eco*RI, B: *Bam*HI) are indicated. Large arrows represent the ORFs of the potential genes, small arrows correspond to the position of oligonucleotides used for RT-PCR experiments (RT1–RT4) and generation of the *pduC*-internal probe for Southern hybridisation (S1 and S2). Sequences between the ORF are shown with the start codon (in bold), stop codon (in italic) and putative RBS (underlined). B: Promotor region between *pocR* and *pduA* genes; the arrows indicate location of the imperfect inverted repeats.

calculated molecular mass of 61, 24.7 and 19.1 kDa, respectively. Analysis of these sequences gave evidence for a putative consensus ribosome binding site sequence (RBS) (GGAGG) located between 8 and 10 bp upstream of the putative initiation codon of each ORF. The calculated molecular mass of the native protein (209 kDa), with a $\alpha_2\beta_2\gamma_2$ composition, was in accordance with that usually found for other dehydratases [3].

The deduced amino acid sequences of the three ORFs (*pduCDE*) were compared with that of diol and glycerol dehydratases from other micro-organisms (Table 1). The large subunit of the *L. collinoides* protein (PduC) exhibited at least 62.2% identity with the corresponding subunit of the proteins of *K. oxytoca*, *S. enterica* and *K. pneumoniae*. PduD and PduE of *L. collinoides* exhibited also significant homologies with the medium and the small subunits, respectively, of diol dehydratases from *K. oxytoca*, *K. pneumoniae* and *S. enterica* (identities of at least 54.3% for the β subunit and of 40.6% for the γ subunit). Homologies were less significant with glycerol dehydratases.

Table 1
Characteristics of homologies between different diol and glycerol dehydratases

Organisms	Gene name			Protein molecular mass (kDa) and number of AA			Amino acid identity (%)		
	α	β	γ	α	β	γ	α	β	γ
L. collinoides	*pduC*	*pduD*	*pduE*	61.0 (558)	24.7 (230)	19.1 (172)	100	100	100
K. oxytoca	*pddA*	*pddB*	*pddC*	60.3 (554)	24.1 (224)	19.1 (173)	62.7	56.1	44.3
S. enterica	*pduC*	*pduD*	*pduE*	60.3 (554)	24.1 (224)	19.1 (173)	62.4	54.3	43.8
K. pneumoniae	*pddA*	*pddB*	*pddC*	60.3 (554)	24.3 (227)	19.4 (174)	62.2	55.7	40.6
C. pasteurianum	*dhaB*	*dhaC*	*dhaE*	60.8 (554)	19.5 (179)	16.7 (146)	61.1	44.8	37
C. freundii	*dhaB*	*dhaC*	*dhaE*	60.4 (555)	21.4 (194)	16.1 (142)	58.4	42.9	34.5
K. pneumoniae	*gldA*	*gldB*	*gldC*	60.6 (555)	21.3 (194)	16.0 (141)	58.2	43.3	33.5

The *pddABC* genes of *K. oxytoca* and *K. pneumoniae* and the *pduCDE* genes encode a diol dehydratase; the *dhaBCE* genes of *C. pasteurianum* and *C. freundii* and the *gldABC* genes encode a glycerol dehydratase. α: Large subunit; β: medium subunit; γ: small subunit. AA: amino acids.

Table 2
Homologies of PduA, PduB and PduG of *L. collinoides* with other proteins in databases

L. collinoides proteins	Protein name	Amino acid identity (%)	Function	Organisms
PduA	PduA	79.4	PD utilisation protein A	*S. enterica*
	PduJ	75.3	PD utilisation protein J	*S. enterica*
PduB	PduB	66.8	PD utilisation protein B	*S. enterica*
PduG	DrdA	66.2	Diol dehydratase-reactivating factor large subunit	*K. oxytoca*
	PduG	65.8	Diol dehydratase-reactivating factor large subunit	*S. enterica*
	OrfZ	62.4	Glycerol dehydratase-reactivating factor large subunit	*C. pasteurianum*
	DhaF	58.3	Glycerol dehydratase-reactivating factor large subunit	*C. freundii*

3.2. Characterisation of the surrounding ORFs

The *pduCDE* genes of *L. collinoides* are surrounded by two ORFs in the upstream region (*pduAB*) and at least two ORFs in the downstream region (*pduGH*). The deduced amino acid sequences from *pduA* and *pduB* exhibited extensive homology with the corresponding genes of *S. enterica* (Table 2).

PduG, located 15 bp downstream of the stop codon of the ORF *pduE*, could encode a large subunit of the reactivating factor for inactivated diol dehydratase. One putative RBS (AGGGG) was found 8 bp upstream of the start codon of the *pduG* gene. Following *pduG*, the beginning of an ORF named *pduH* was sequenced. No homology was found regarding this gene.

A 948-bp ORF was located upstream of the putative *pdu* operon and transcribed divergently. This gene (*pocR*) could encode a 317-amino acid protein which exhibited 37% identity over 110 residues in the C-terminal extremity (18% over the entire protein) with the corresponding protein of *S. enterica*, the regulator of the *pdu* operon [5].

In order to determine if the diol dehydratase genes were transcribed together with the *pduAB* genes, reverse transcriptase experiments were realised with cDNA obtained from *pduC* (Fig. 2). The data showed that *pduAB* and the diol dehydratase genes constitute effectively an operon. This result suggests that *pduCDE* are regulated via the 346-bp region upstream of *pduA* (Fig. 1B). Analysis of this region exhibited an average G+C of 30% in comparison to 45% normally found in the chromosome of *L. collinoides*. Three imperfect inverted repeats with the following consensus TTTtAAAnTAATTggaA are present in this sequence which may be the recognition sequences of PocR as previously shown in *S. enterica* [5].

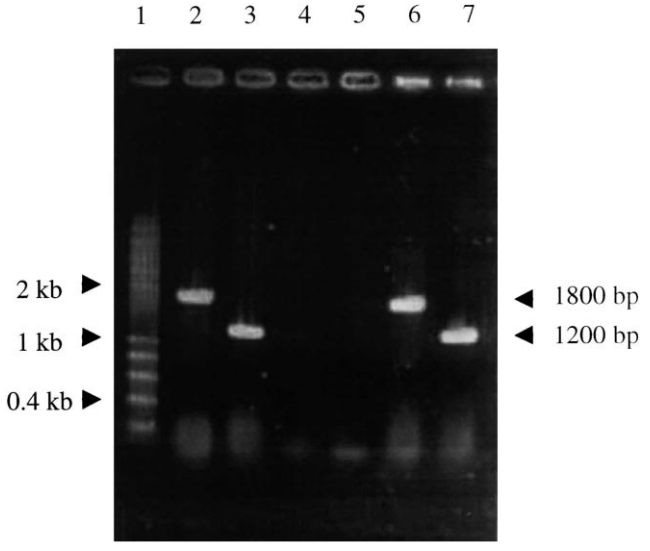

Fig. 2. Reverse transcriptase PCR assays performed with RT4–RT2 (*pduA–pduC*) for lanes 2, 4, 6; RT3–RT2 (*pduB–pduC*) for lanes 3, 5, 7; negative control were performed without reverse transcriptase (lanes 4, 5); positive control were performed with genomic DNA (lanes 6, 7); lane 1: molecular mass marker (Smartladder, Eurogentec).

3.3. Southern blot analysis

Fig. 3 shows the Southern hybridisation analysis of digested chromosomal DNA. The 3′ *pduC* extremity, highly conserved between glycerol or diol dehydratases, was used as probe. The result strongly suggests that *L. collinoides* contains only the diol dehydratase operon.

3.4. Expression of the diol dehydratase activity

The activity of the dehydratase measured in *L. collinoides* grown under different conditions is presented in Fig. 4A. Dehydratase activity was detected when glucose was used as carbon source. This activity increased during the exponential growth phase before being stable in stationary phase (0.36 U mg^{-1} protein). Co-fermentation with 1,2-PD resulted in a two-fold higher activity (average 0.6 U mg^{-1} protein). This positive effect was not observed with glycerol, and the enzyme activity dropped at the beginning of the stationary phase. This decrease may result from the lethal effect of the 3-HPA accumulation on *L. collinoides* (Fig. 4B) as survival effectively decreased under this condition (data not shown). No significant activity was evidenced when *L. collinoides* was grown on arabinose or arabinose plus 1,2-PD.

4. Discussion

L. collinoides, a micro-organism currently encountered in cider, is correlated with an alteration known as 'piqûre acroléique' involving glycerol degradation. Like all bacte-

ria of the same genera using glycerol, *L. collinoides* does not carry the oxidative pathway and uses it via a system in which the first enzyme is a dehydratase. The entire sequence of the genes encoding this enzyme strongly suggests that glycerol is not the privileged substrate. Indeed, the degrees of homologies observed with other dehydratases, the molecular mass deduced from the ORFs as well as the genetic environment of these genes showed that this protein is rather a diol dehydratase than a glycerol dehydratase. Therefore, the name previously used to design the gene, *dha* [10], was not relevant and we proposed to rename these genes *pdu* for *p*ropane*d*iol *u*tilisation.

In *L. collinoides*, this enzyme is supposed to have a traditional structure with three subunits of α, β and γ, suggesting an hexamer complex [3]. This is in contrast with the structure proposed in *L. reuteri* by Talarico [7]. The genes of the enzyme diol dehydratase are part of a putative operon of at least seven genes. The highest identities were found with *K. oxytoca*. However, it was surprising to discover similarities with the 5′ extremity of the *pdu* operon of *S. enterica*. Two genes located upstream of the genes of the diol dehydratase were hitherto only found in *S. enterica* LT2, suggesting a common ancestor. They encode proteins related to the formation of polyhedral bodies which confine the dehydratase and limit the toxic 3-HPA diffusion out of the complex or protect the enzyme from oxygen [3]. In *Salmonella*, this pathway allows growth via the anaerobic respiration of 1,2-PD. As *L. collinoides* can not grow on PD as sole carbon and energy source, it can be speculated that this bacterium uses this pathway only for NADH regeneration.

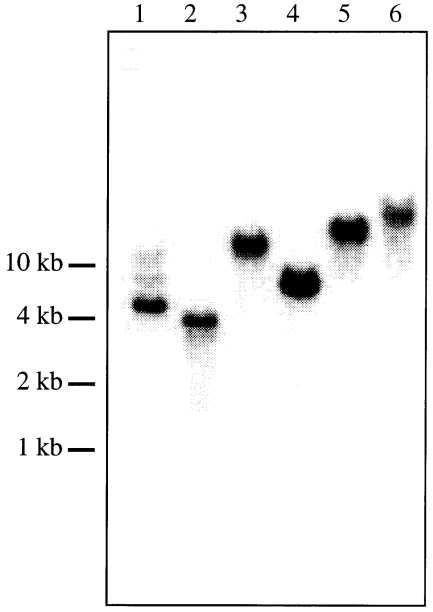

Fig. 3. Southern hybridisation analysis using an internal sequence of *pduC* as probe. Genomic DNA of *L. collinoides* was digested with *Bam*HI (lane 1); *Cla*I (lane 2); *Eco*RV (lane 3); *Hin*dIII (lane 4); *Pst*I (lane 5) and *Xba*I (lane 6).

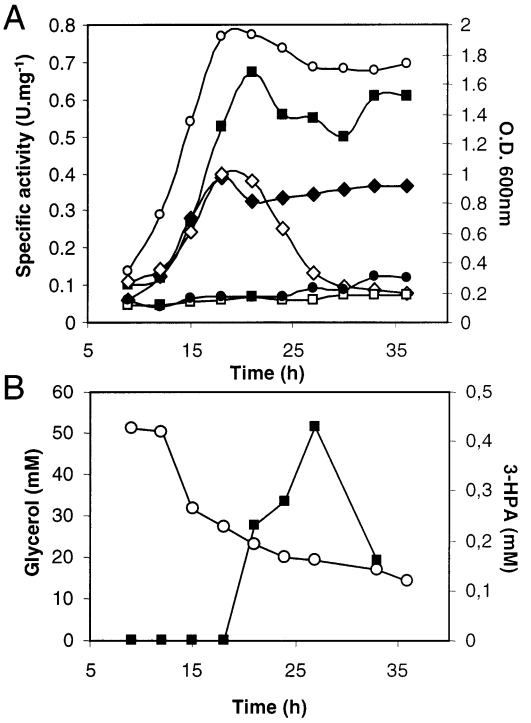

Fig. 4. A: Growth of *L. collinoides* (○) and specific activity of the diol dehydratase on MRS medium containing 15 mM glucose (♦), 15 mM glucose and 54 mM glycerol (◇), 15 mM glucose and 65 mM 1,2-PD (■), 20 mM arabinose (□), 20 mm arabinose and 65 mM 1,2-PD (●). B: Time course of glycerol (○) and 3-HPA (■) concentrations.

The dehydratase activity detected in *L. collinoides* grown on various carbon sources shows that this pathway is used when glucose is the energy source. The increase in activity was inversely proportional to the remaining glucose concentration in the medium, suggesting a potential regulation of the enzyme. Moreover, unlike glycerol, 1,2-PD seems to have a positive effect on the protein activity or expression in late exponential phase (18 h). Finally, as previously described on ribose [4], no activity was found in *L. collinoides* cells grown on the pentose arabinose, independently of the culture conditions.

Thus, the expression of the enzyme on medium containing glucose and its ability to degrade glycerol, conditions encountered in cider at the end of the fermentation, may lead to the 'piqûre acroléique' alteration by *L. collinoides*.

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